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(57) Abstract

Primers designed to be immobilized on microspheres are allowed to anneal to the DNA strand under investigation, and are extended by either DNA polymerase using fluorescent dideoxynucleotides or ligated by DNA ligase to fluorescent reporter oligonucleotides. The fluorescence of either the dideoxynucleotide or the reporter oligonucleotide attached to the immobilized primer is measured by flow cytometry, thereby identifying the nucleotide polymorphism on the DNA strand.

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DNA POLYMORPHISM IDENTITY DETERMINATION USING FLOW CYTOMETRY

The present patent application claims priority from Provisional Patent Application No. 60/063,685, which was filed on October 28, 1997.

FIELD OF THE INVENTION

The present invention relates generally to the use of flow cytometry for the determination of DNA nucleotide base composition and, more particularly, to the use flow cytometry to determine the base identification of single nucleotide polymorphisms, including nucleotide polymorphisms, insertions, and deletions. This invention was made with government support under Contract No. W-7405-ENG-36 awarded by the US Department of Energy to The Regents of The University of California. The government has certain rights in this invention.

BACKGROUND OF THE INVENTION

The determination of the DNA base sequence of the human genome will have a major impact on biomedical science in the next century. The completion of the first complete human DNA will enhance a range of applications from genetic mapping of disease-associated genes to diagnostic tests for disease susceptibility and drug response. The determination of base composition at specific, variable DNA sites known as single nucleotide polymorphisms (SNPs) is especially important. The current generation of sequence determination methods are too slow and costly to meet large-scale SNP analysis requirements. Thus, there is a need for faster, more efficient methods for analyzing genetic sequences for SNPs.

SNPs have a number of uses in mapping, disease gene identification, and diagnostic assays. All of these applications involve the determination of base composition at the SNP site. Conventional sequencing can provide this information, but is impractical for screening a large number of sites in a large number of individuals. Several alternative methods have been developed to increase throughput.

Two techniques have been developed to determine base composition at a single

site, minisequencing (See, e.g., "Minisequencing: A Specific Tool For DNA Analysis And Diagnostics On Oligonucleotide Arrays," by Tomi Pastinen et al., Genome Research **7**, 606 (1997)), and oligo-ligation (See, e.g., "Single-Well Genotyping Of Diallelic Sequence Variations By A Two-Color ELISA-Based Oligonucleotide Ligation Assay," by Vincent O. Tobe et al., Nuclear Acids Res. **24**, 3728 (1996)). In minisequencing, a primer is designed to interrogate a specific site on a sample template, and polymerase is used to extend the primer with a labeled dideoxynucleotide. In oligo-ligation, a similar primer is designed, and ligase is used to covalently attach a downstream oligo that is variable at the site of interest. In each case, the preference of an enzyme for correctly base-paired substrates is used to discriminate the base identity that is revealed by the covalent attachment of a label to the primer. In most applications these assays are configured with the primer immobilized on a solid substrate, including microplates, magnetic beads and recently, oligonucleotides microarrayed on microscope slides. Detection strategies include direct labeling with fluorescence detection or indirect labeling using biotin and a labeled streptavidin with fluorescent, chemiluminescent, or absorbance detection.

Oligonucleotide microarrays or "DNA chips" have generated much attention for their potential for massively parallel analysis. The prospect of sequencing tens of thousands of bases of a small sample in just a few minutes is exciting. At present, this technology has limited availability in that arrays to sequence only a handful of genes are currently available, with substantial hardware and consumable costs. In addition, the general approach of sequencing by hybridization is not particularly robust, with the requirement of significant sequence-dependent optimization of hybridization conditions. Nonetheless, the parallelism of an "array" technology is very powerful. and multiplexed sequence determination is an important element of the new flow cytometry method.

Accordingly, it is an object of the present invention to provide a method for determining the base composition at specific sites in a strand of DNA using microspheres and flow cytometry, wherein the specificity of enzymes for discriminating base composition is combined with the parallel analysis of a fluorescent microsphere

array.

Additional objects, advantages, and novel features of the invention will be set forth in part in the description which follows, and in part will become apparent to those skilled in the art upon examinations of the following or may be learned by practice of the invention. The objects and advantages of the invention may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims.

SUMMARY OF THE INVENTION

To achieve the forgoing and other objects, and in accordance with the purposes of the present invention as embodied and broadly described herein, the method for determining the base composition at specific sites on a DNA strand hereof includes the steps of: preparing an oligonucleotide primer bearing an immobilization or capture tag, fluorescently labeled dideoxynucleotides; extending the oligonucleotide primer using DNA polymerase with the fluorescent dideoxynucleotide; specifically binding the tagged primers to microspheres; and measuring microsphere fluorescence by flow cytometry.

Preferably, the oligonucleotide primers are designed to anneal to the DNA sample under investigation immediately adjacent to the site of interest so as to interrogate the next nucleotide base on the DNA sample.

It is also preferred that the primers have on their 5' terminus one of: (a) an amino or other functional group suitable for covalent coupling to a microsphere; (b) a biotin group suitable for binding to avidin or streptavidin immobilized on a microsphere; or (c) an oligonucleotide tag that is complementary to an oligonucleotide capture probe immobilized on a microsphere surface.

In another aspect of the present invention, in accordance with its objects and purposes, the method for determining the base composition at specific sites on a DNA strand hereof includes the steps of: preparing an oligonucleotide primer bearing an immobilization or capture tag, fluorescently labeled dideoxynucleotides; preparing a fluorescent reporter oligonucleotide; enzymatically ligating the oligonucleotide primer to the fluorescent reporter oligonucleotide; specifically binding the tagged primers to

microspheres; and measuring microsphere fluorescence by flow cytometry.

Benefits and advantages of the present invention include a sensitive, homogenous, and flexible method for determining DNA base composition at specific sites.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and form part of the specification, illustrate an embodiment of the present invention and, together with the description, serve to explain the principles of the invention. In the drawings:

FIGURE 1a is a schematic representation of microsphere-based minisequencing for flow cytometry, where a primer immobilized on a microsphere is used for hybridizing with the DNA sequence under investigation in the presence of dideoxynucleotides, at least one of which is fluorescently labeled, and polymerase, whereby the primer is extended by one base, while FIG. 1b is a schematic representation of the resulting primer having a single, fluorescent dideoxynucleotide bound to the end thereof which can be detected using flow cytometry, and represents the complementary base to the SNP on the DNA.

FIGURE 2a is a schematic representation of microsphere-based minisequencing for flow cytometry similar to that described in FIGS. 1a and 1b hereof, except that soluble biotinylated primers and avidin-coated capture microspheres are used instead of primers which have already been immobilized on the microspheres, FIGURE 2b shows the hybridization of the biotinylated primer to the DNA strand to be investigated and the extension of this primer by a fluorescent A dideoxynucleotide (assuming that the SNP is a T base) as a result of the DNA polymerase present in the solution, and FIGURE 2c shows the capture of the extended biotinylated primer onto an avidin-coated microsphere after the hybridized DNA strand is melted, with the subsequent fluorescence analysis using flow cytometry.

FIGURE 3a is a schematic representation of a multiplexed microsphere-based minisequencing procedure using soluble sequence-tagged primers and capture probe-bearing microspheres in a similar manner to the minisequencing illustrated in FIGS. 2a

and 2b hereof, except that four SNPs have been assumed to be present on the DNA strand, while FIGURE 3b illustrates the microspheres and the captured extended primers to be analyzed using flow cytometry.

FIGURE 4a is a schematic representation of microsphere-based oligonucleotide ligation assay using flow cytometry, where a primer immobilized on a microsphere along with fluorescent complementary primers for ligating to the primer which has hybridized to the DNA strand to be investigated in the region of the SNP, while FIGURE 4b is a schematic representation of the microsphere-attached primer to which the proper fluorescent complement has been ligated after the DNA has been melted away, the flow cytometric determined fluorescence of the microsphere indicating which fluorescent complement has been attached to the DNA strand.

FIGURES 5a and 5b are schematic representations of oligonucleotide ligation on unamplified DNA, followed by PCR amplification, capture on microspheres, and analysis of microsphere fluorescence by flow cytometry, for the case where the complementary base is found on the DNA strand and where the complementary base does not exist on the DNA strand, respectively.

DETAILED DESCRIPTION

Briefly, the present invention includes the use of oligonucleotide primers, fluorescent dideoxynucleotides, DNA polymerase, microspheres, and flow cytometry to determine DNA base composition at specific sites in a DNA strand. Tagged oligonucleotide primers are incubated with a DNA sample and allowed to anneal immediately adjacent to the site of interest. Fluorescent dideoxynucleotides and DNA polymerase are added and allowed to extend the primer by one base unit, such that upon enzymatic incorporation of the single fluorescent dideoxynucleotide into the DNA strand, the DNA strand can be detected by a flow cytometer. DNA polymerase may be Sequenase, Thermosequenase, or any other conventional or thermostable DNA polymerase.

Another embodiment of the invention uses oligonucleotide primers, oligonucleotide reporters and DNA ligase along with microspheres and flow cytometry to

make this determination. A fluorescent reporter oligonucleotide and DNA ligase are added and allowed to ligate the primer to the reporter. The fluorescent reporter oligonucleotides are designed to bind the sample DNA immediately 3' to the annealed oligonucleotide primer. That is, the sequence reporter oligonucleotide is complementary to that of the sample DNA strand except at its 5' terminus, where the reporter is variable so as to interrogate the site of interest on the sample DNA, which can then be investigated by its fluorescent signature using flow cytometry. The DNA ligase may be any conventional or thermostable ligase. Primer extension or ligation may be enhanced through the use of thermal cycling using heat-stable DNA polymerase or ligase.

Oligonucleotide primers are bound to microspheres either before or after enzymatic extension or ligation. Amino-labeled primers can be covalently attached to carboxylated microspheres using EDAC. Biotinylated primers can be attached to avidin or streptavidin-coated microspheres. Primers bearing an oligonucleotide sequence tag may be annealed to complementary oligonucleotide capture sequences immobilized on microspheres covalently or by the biotin-avidin interaction. Microspheres may be composed of polystyrene, cellulose, or other appropriate material. Microspheres having different sizes, or stained with different amounts of fluorescent dyes, may be used to perform multiplexed sequence analysis.

Having generally described the invention, the following EXAMPLES are intended to provide more specific details thereof.

EXAMPLE 1

Flow Cytometric Minisequencing Using Immobilized Primers:

Reference will now be made in detail to the preferred embodiments of the present invention as illustrated in the accompanying drawings. Turning now to the Figures, Fig. 1a is a schematic representation of microsphere-based minisequencing for flow cytometry, where a primer immobilized on a microsphere is used for hybridizing with the DNA sequence under investigation in the presence of dideoxynucleotides, at least one of which is fluorescently labeled, and polymerase. The primer is extended by

one base by the action of the polymerase. Figure 1b is a schematic representation of the resulting primer having a single, fluorescent dideoxynucleotide bound to the end thereof which can be detected using flow cytometry, and represents the complementary base to the SNP on the DNA. The sample DNA template is first amplified using the polymerase chain reaction (PCR), and the resulting product treated with shrimp alkaline phosphatase (SAP) and exonuclease I (Exo I) to remove unconsumed deoxynucleotide triphosphates and PCR primers, respectively. The minisequencing primer, designed to interrogate a specific site on the DNA strand under investigation, is immobilized by means of a 5'-amino group on a carboxylated polystyrene microsphere using a cross-linking reagent (e.g., carbodiimide). The primer-bearing microspheres (5 μl) are added to the amplified DNA (1 μl, 1 nM) DNA polymerase (one unit, Thermosequenase, Amersham Life Sciences, Cleveland, OH), one fluorescein-labeled ddNTP (5μM), 5 μM each of the other three non-fluorescent ddNTPs, and buffer (Thermosequenase buffer, Amersham) in a total volume of 10 μl. This process was repeated three times using each of the four fluorescent ddNTPs. The reaction mixtures are cycled 99 times at 94 °C for 10 s and at 60 °C for 10 s in a thermal cycler. Two microliters of each reaction mixture were diluted into 500 μl of TEB buffer (50 mM Tris-HCl, pH, 8.0, 0.5 mM EDTA, 0.5% (w/v) bovine serum albumin, BSA), and the microsphere-associated fluorescence was measured using flow cytometry. Using this procedure, the correct nucleotide base identity was determined for a specific position on an oligonucleotide template with a signal-to-background ratio of greater than one hundred.

EXAMPLE 2

Flow Cytometric Minisequencing Using Biotinylated Primers:

Figure 2a is a schematic representation of microsphere-based minisequencing for flow cytometry similar to that described in Figs. 1a and 1b hereof, except that soluble biotinylated primers and avidin-coated capture microspheres are used instead of primers which have already been immobilized on the microspheres. Figure 2b shows the hybridization of the biotinylated primer to the DNA strand to be investigated and the extension of this primer by a single, fluorescent A dideoxynucleotide (assuming that the

SNP is a T base) as a result of the DNA polymerase present in the solution. Figure 2c shows the capture of the extended biotinylated primer onto an avidin-coated microsphere after the hybridized DNA strand is melted, with the subsequent fluorescence analysis using flow cytometry. The sample DNA template is amplified by PCR, and the resulting product treated with shrimp alkaline phosphatase (SAP) and exonuclease I (Exo I) to remove unconsumed deoxynucleotide triphosphates and PCR primers, respectively. The minisequencing primer, designed to interrogate a specific site on the template DNA, and bearing a 5'-biotin group, is prepared. The biotinylated primer is added to the template DNA (1 μl, 1 nM), DNA polymerase (one unit, Thermosequenase, Amersham), one fluorescein-labeled ddNTP (5 μM), 5 μM each of the other three non-fluorescent ddNTPs, and buffer (Thermosequenase buffer, Amersham) in a total volume of 10 μl. This process is repeated three times using a different fluorescent ddNTP. The reaction mixtures are cycled 99 times at 94 °C for 10 s and 60 °C for 10 s in a thermal cycler. Five μl of avidin-coated microspheres are added to the reaction mixture to capture the biotinylated primers. Two microliters of each reaction mixture is diluted into 500 μl of TEB buffer (50 mM Tris-HCl, pH, 8.0, 0.5 mM EDTA, 0.5% (w/v) bovine serum albumin, BSA), and the microsphere-associated fluorescence is measured using flow cytometry. Using this procedure, the correct nucleotide base identity was determined in thirty out of thirty PCR amplified samples as was confirmed by conventional DNA sequencing techniques.

EXAMPLE 3

Flow Cytometric Minisequencing Using Tagged Primers:

Figure 3a is a schematic representation of a multiplexed microsphere-based minisequencing procedure using soluble sequence-tagged primers and capture probe-bearing microspheres in a similar manner to the minisequencing illustrated in Figs. 2a and 2b hereof, except that four SNPs have been assumed to be present on the DNA strand. Figure 3b illustrates the microspheres and the captured extended primers to be analyzed using flow cytometry. The sample DNA template is amplified by PCR, and the resulting product treated with shrimp alkaline phosphatase (SAP) and exonuclease I

(Exo I) to remove unconsumed deoxynucleotide triphosphates and PCR primers, respectively. The minisequencing primer, designed to interrogate a specific site on the template DNA, and bearing a 5'-sequence tag is prepared. A capture probe is designed to bind to the 5'-sequence tag of the primer, and is immobilized on microspheres. The capture tag-bearing primer is added to the template DNA (1 μl, 1 nM), DNA polymerase (one unit, Thermosequenase, Amersham), one fluorescein-labeled ddNTP (5 μM), 5 μM of each of the other three non-fluorescent ddNTPs, and buffer (Thermosequenase buffer, Amersham) in a total volume of 10 μl. This process is repeated three times using a different fluorescent ddNTP. The reaction mixtures are cycled 99 times at 94 °C for 10 s and 60 °C for 10 s in a thermal cycler. Five microliters of avidin-coated microspheres are added to the reaction mixture to capture the biotinylated primers. Two microliters of each reaction mixture is diluted into 500 μl of TEB buffer (50 mM Tris-HCl, pH, 8.0, 0.5 mM EDTA, 0.5% (w/v) bovine serum albumin, BSA), and the microsphere-associated fluorescence is measured using flow cytometry.

EXAMPLE 4

Flow Cytometric Oligonucleotide Ligation Using Immobilized Primers:

Figure 4a is a schematic representation of microsphere-based oligonucleotide ligation assay using flow cytometry, where a primer immobilized on a microsphere along with fluorescent complementary primers for ligating to the primer which has hybridized to the DNA strand to be investigated in the region of the SNP. Figure 4b is a schematic representation of the microsphere-attached primer to which the proper fluorescent complement has been ligated after the DNA has been melted away, the flow cytometric determined fluorescence of the microsphere indicating which fluorescent complement has been attached to the DNA strand. The sample DNA template is amplified by PCR, and the resulting product treated with shrimp alkaline phosphatase (SAP) and exonuclease I (Exo I) to remove unconsumed deoxynucleotide triphosphates and PCR primers, respectively. The oligonucleotide ligation primer, designed to interrogate a specific site on the template DNA, is immobilized via a 5'-amino group on a carboxylated polystyrene microsphere using carbodiimide. Four fluorescent reporter

oligonucleotides designed to bind immediately adjacent to the site of interest, but varying at the 5'-terminus are prepared. The primer-bearing microspheres (5 μl) are added to the template DNA (1 μl, 1 nM), DNA ligase (one unit, Thermoligase, Epicentre Technologies, Madison, WI), one fluorescein-labeled reporter oligonucleotide (5 μM), and buffer (Thermoligase buffer, Epicentre) in a total volume of 10 μl. This process is repeated three times using each of the four fluorescent reporter oligonucleotides (5 μM). The reaction mixtures are cycled 99 times at 94 °C for 10 s and 60 °C for 10 s in a thermal cycler. Two microliters of each reaction mixture are diluted into 500 μl of TEB buffer (50 mM Tris-HCl, pH, 8.0, 0.5 mM EDTA, 0.5% (w/v) bovine serum albumin, BSA), and the microsphere-associated fluorescence is measured using flow cytometry. Using this procedure, the correct nucleotide base identity was determined in thirty out of thirty PCR amplified samples as was confirmed by conventional DNA sequencing techniques.

EXAMPLE 5

Multiplexed Oligonucleotide Ligation On Unamplified DNA, Followed By PCR Amplification:

Figures 5a and 5b are schematic representations of oligonucleotide ligation on unamplified DNA, followed by PCR amplification, capture on microspheres, and analysis of microsphere fluorescence by flow cytometry, for the case where the complementary base is found on the DNA strand and where the complementary base does not exist on the DNA strand, respectively.

A set of oligonucleotide primers is designed including one oligonucleotide (oligonucleotide 1) that is complementary to the sequence of the template DNA immediately adjacent to a site of interest, and four oligonucleotides (oligonucleotides 1A, 1C, 1G, and 1T) that are complementary to the sequence of the template DNA immediately adjacent to oligonucleotide 1, and containing the site of interest. Each of the four oligonucleotides (1A, 1C, 1G, and 1T) differs in the nucleotide base adjacent to the other oligonucleotide (oligonucleotide 1), corresponding to each of the four possible bases, A, C, G, and T. Oligonucleotide 1 is intended to ligate to one of the other

oligonucleotides (1A, 1C, 1G, or 1T), depending which one contains the complementary base for the site of interest. In addition, each of the two oligonucleotides in a potential pair (five oligonucleotides total) contain additional nucleotides that form a "tail" consisting of a PCR priming site. This site is different for oligonucleotide 1 than for oligonucleotides 1A, 1C, 1G, and 1T, which have the same primer-binding site within this group, but different from that of oligonucleotide 1. Four parallel ligation reactions are performed, each with oligonucleotide 1, one each of the other oligonucleotides (1A, 1C, 1G, or 1T) and a DNA ligase enzyme. All oligonucleotides are expected to hybridize to the template, but only the oligonucleotide with a perfect match will be ligated to oligonucleotide 1. The resulting ligation product will serve as the template for a PCR reaction that follows using one primer (primer 1) complementary to the tail introduced into oligonucleotide 1, and the other primer (primer 2) having the same sequence as that of the tail of oligonucleotides 1A, 1C, 1G, and 1T. Unligated oligonucleotides cannot be amplified with the PCR technique (Fig. 5b) because there is no priming site for oligonucleotide 2 unless PCR amplification from primer 1 extends across a ligated fragment, creating sequence complementary to primer 2. In addition to unlabeled dNTPs used during the PCR step, fluorescently labeled dNTPs are added to label the PCR fragments during amplification. Alternatively, primer 2 is labeled with a fluorescent dye, producing dye-labeled PCR amplification products where amplification occurs.

The final step involves adding to the PCR mixture microspheres with an oligonucleotide immobilized on its surface that has the same sequence as oligonucleotides 1A, 1C, 1G, and 1T, except for the variable nucleotide at one end and the priming site on the other. This microsphere is intended to capture labeled PCR products if they are present in the PCR mixture by annealing to the newly synthesized complement of the ligated oligonucleotide complex. Bead fluorescence due to hybridized fragments is then analyzed by flow cytometry. Many sets of primers can simultaneously type many SNPs in solution, each being captured onto a different bead in a multiplexed set to be simultaneously read in a flow cytometer.

The foregoing description of the invention has been presented for purposes of illustration and description and is not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teaching. For example, in order to bind the oligonucleotide primers to the microspheres for analysis using flow cytometry, the oligonucleotide primers may include a sequence tag which is hybridized to a capture probe that is complementary to the sequence tag and is immobilized on the microspheres, the sequence tags and capture probes containing at least one of the non-natural bases iso-C and 5-methyl-iso-G. The embodiments were chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.

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6. The method for determining the base identity at specific sites on a DNA strand as described in claim 1, wherein the oligonucleotide primers are hybridized to a complementary capture probe immobilized on microspheres.

7. The method for determining the base identity at specific sites on a DNA strand as described in claim 1, wherein the oligonucleotide primers include a sequence tag which is hybridized to a capture probe that is complementary thereto and is immobilized on the microspheres, and wherein the sequence tags and capture probes contain at least one of the non-natural bases iso-C and 5-methyl-iso-G.

8. A method for determining the base identity at specific sites on a DNA strand, which comprises the steps of:

(a) synthesizing an oligonucleotide primer which can anneal to the DNA strand immediately adjacent to the base under investigation;

(b) immobilizing the oligonucleotide primer on microspheres;

(c) annealing the oligonucleotide primer to the DNA strand;

(d) incubating the microspheres to which the DNA strand has annealed to the immobilized oligonucleotide primer with fluorescent reporter molecules in the presence of an enzyme, each of the fluorescent reporter molecules having a labile base, whereby a fluorescent reporter molecule having a labile complementary nucleotide base to the base under investigation covalently attaches to the immobilized oligonucleotide primer;

(e) melting the DNA strand off of the ligated oligonucleotide; and

(f) analyzing the fluorescence of the microspheres using flow cytometry.

9. The method for determining the base identity at specific sites on a DNA strand as described in claim 8, wherein the reporter molecule includes fluorescent oligonucleotides and the enzyme includes DNA ligase.

10. The method for determining the base identity at specific sites on a DNA strand as described in claim 8, wherein the oligonucleotide primers are biotinylated and the microspheres are coated with avidin.

11. The method for determining the base identity at specific sites on a DNA

strand as described in claim 8, wherein the oligonucleotide primers are biotinylated and the microspheres are coated with streptavidin.

12. The method for determining the base identity at specific sites on a DNA strand as described in claim 8, wherein the oligonucleotide primers are covalently attached to the microspheres.

13. The method for determining the base identity at specific sites on a DNA strand as described in claim 8, wherein the oligonucleotide primers are hybridized to a complementary capture probe immobilized on microspheres.

14. The method for determining the base identity at specific sites on a DNA strand as described in claim 8, wherein the oligonucleotide primers include a sequence tag which is hybridized to a capture probe that is complementary thereto and is immobilized on the microspheres, and wherein the sequence tags and capture probes contain at least one of the non-natural bases iso-C and 5-methyl-iso-G.

15. A method for determining the base identity at specific sites on a DNA strand, which comprises the steps of:

(a) synthesizing an oligonucleotide primer which can anneal to the DNA strand immediately adjacent to the base under investigation;

(b) annealing the oligonucleotide primer to the DNA strand;

(c) incubating the annealed DNA strand and oligonucleotide primer with fluorescent reporter molecules in the presence of an enzyme, each of the fluorescent reporter molecules having a labile oligonucleotide base, whereby a fluorescent reporter molecule having a labile complementary base to the oligonucleotide base under investigation covalently attaches to the oligonucleotide primer extending the oligonucleotide primer by one base unit;

(d) immobilizing the extended oligonucleotide primers on microspheres; and

(e) analyzing the fluorescence of the microspheres using flow cytometry.

16. The method for determining the base identity at specific sites on a DNA

strand as described in claim 15, wherein the fluorescent reporter molecules include fluorescent dideoxynucleotides and the enzyme includes DNA polymerase.

17. The method for determining the base identity at specific sites on a DNA strand as described in claim 15, wherein the oligonucleotide primers are biotinylated and the microspheres are coated with avidin.

18. The method for determining the base identity at specific sites on a DNA strand as described in claim 15, wherein the oligonucleotide primers are biotinylated and the microspheres are coated with streptavidin.

18. The method for determining the base identity at specific sites on a DNA strand as described in claim 15, wherein the oligonucleotide primers are covalently attached to the microspheres.

19. The method for determining the base identity at specific sites on a DNA strand as described in claim 15, wherein the oligonucleotide primers are hybridized to a complementary capture probe immobilized on microspheres.

20. The method for determining the base identity at specific sites on a DNA strand as described in claim 15, wherein the oligonucleotide primers include a sequence tag which is hybridized to a capture probe that is complementary thereto and is immobilized on the microspheres, and wherein the sequence tags and capture probes contain at least one of the non-natural bases iso-C and 5-methyl-iso-G.

21. A method for determining the base identity at specific sites on a DNA strand, which comprises the steps of:

(a) synthesizing an oligonucleotide primer can anneal to the DNA strand immediately adjacent to the base under investigation;

(b) annealing the oligonucleotide primer to the DNA strand;

(c) incubating the annealed DNA strand and oligonucleotide primer with fluorescent reporter molecules in the presence of an enzyme, each of the fluorescent reporter molecules having a labile base, whereby a fluorescent reporter molecule having a labile complementary nucleotide base to the base under investigation covalently attaches to the oligonucleotide primer;

(d) melting the DNA strand off of the ligated oligonucleotide primer;

(e) immobilizing the extended oligonucleotide primers on microspheres; and

(f) analyzing the fluorescence of the microspheres using flow cytometry.

22. The method for determining the base identity at specific sites on a DNA strand as described in claim 21, wherein the reporter molecules includes fluorescent oligonucleotides and the enzyme includes DNA ligase.

23. The method for determining the base identity at specific sites on a DNA strand as described in claim 21, wherein the oligonucleotide primers are biotinylated and the microspheres are coated with avidin.

24. The method for determining the base identity at specific sites on a DNA strand as described in claim 21, wherein the oligonucleotide primers are biotinylated and the microspheres are coated with streptavidin.

25. The method for determining the base identity at specific sites on a DNA strand as described in claim 21, wherein the oligonucleotide primers are covalently attached to the microspheres.

26. The method for determining the base identity at specific sites on a DNA strand as described in claim 21, wherein the oligonucleotide primers are hybridized to a complementary capture probe immobilized on microspheres.

27. The method for determining the base identity at specific sites on a DNA strand as described in claim 21, wherein the oligonucleotide primers include a sequence tag which is hybridized to a capture probe that is complementary thereto and is immobilized on the microspheres, and wherein the sequence tags and capture probes contain at least one of the non-natural bases iso-C and 5-methyl-iso-G.

AMENDED CLAIMS

[received by the International Bureau on 09 March 1999 (09.03.99); new claims 1-5 added; (1 page)]

1. A method for determining the base identity at specific sites on a DNA strand, which comprises the steps of:

(a) synthesizing an oligonucleotide primer which can anneal to the DNA strand immediately adjacent to the base under investigation;

(b) immobilizing the oligonucleotide primer on microspheres;

(c) annealing the oligonucleotide primer to the DNA strand;

(d) incubating the microspheres to which the DNA strand has annealed to the immobilized oligonucleotide primer with fluorescent reporter molecules in the presence of an enzyme, each of the fluorescent reporter molecules having a labile base, whereby a fluorescent reporter molecule having a labile complementary base to the base under investigation covalently attaches to the immobilized oligonucleotide primer extending the immobilized oligonucleotide primer by one base unit; and

(e) analyzing the fluorescence of the microspheres using flow cytometry.

2. The method for determining the base identity at specific sites on a DNA strand as described in claim 1, wherein the fluorescent reporter molecules include fluorescent dideoxynucleotides and the enzyme includes DNA polymerase.

3. The method for determining the base identity at specific sites on a DNA strand as described in claim 1, wherein the oligonucleotide primers are biotinylated and the microspheres are coated with avidin.

4. The method for determining the base identity at specific sites on a DNA strand as described in claim 1, wherein the oligonucleotide primers are biotinylated and the microspheres are coated with streptavidin.

5. The method for determining the base identity at specific sites on a DNA strand as described in claim 1, wherein the oligonucleotide primers are covalently attached to the microspheres.

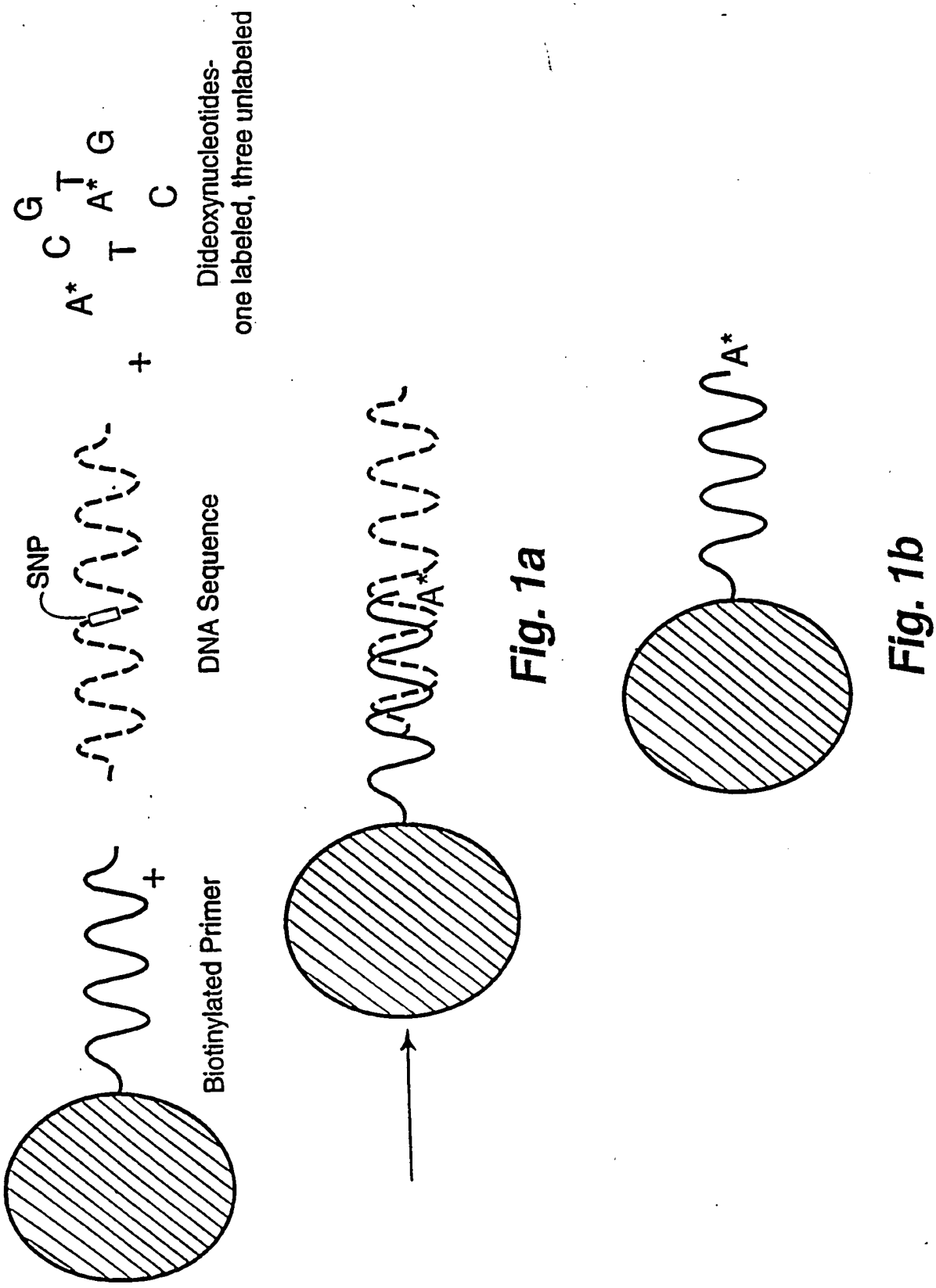

Fig. 1a

Fig. 1b

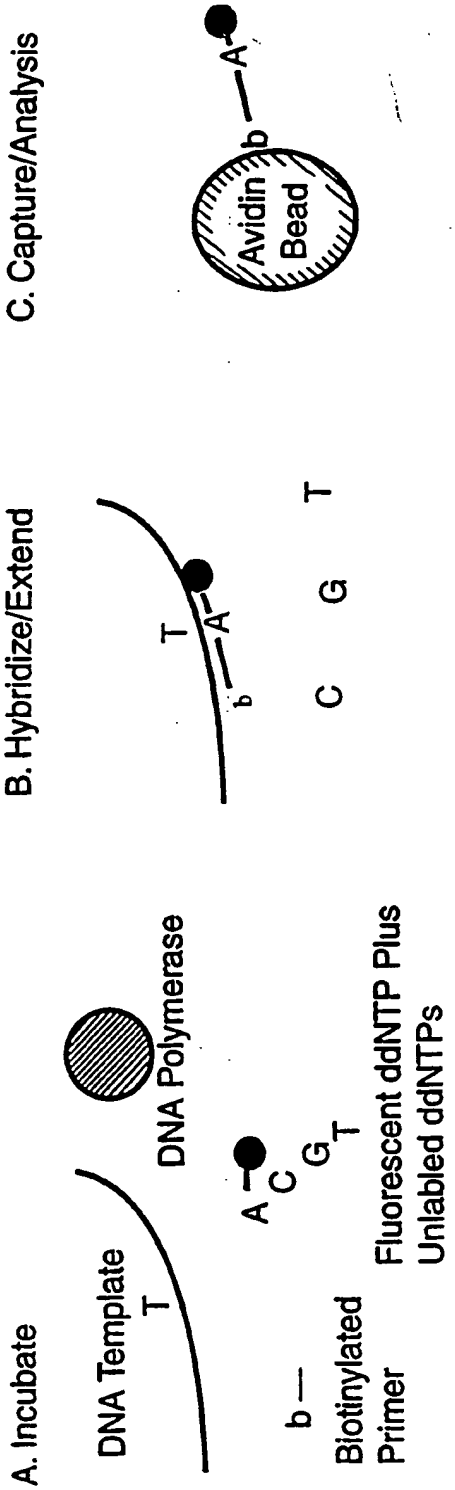

Fig. 2a

Fig. 2b

Fig. 2c

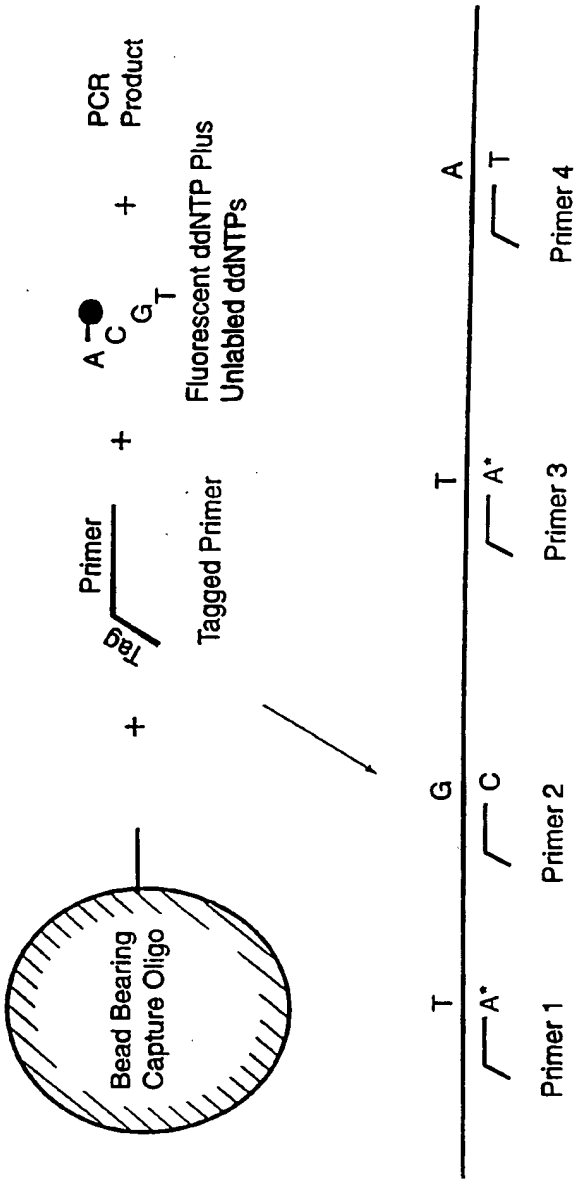

Fig. 3a

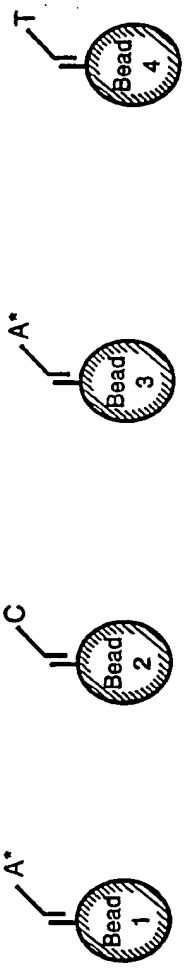

Fig. 3b

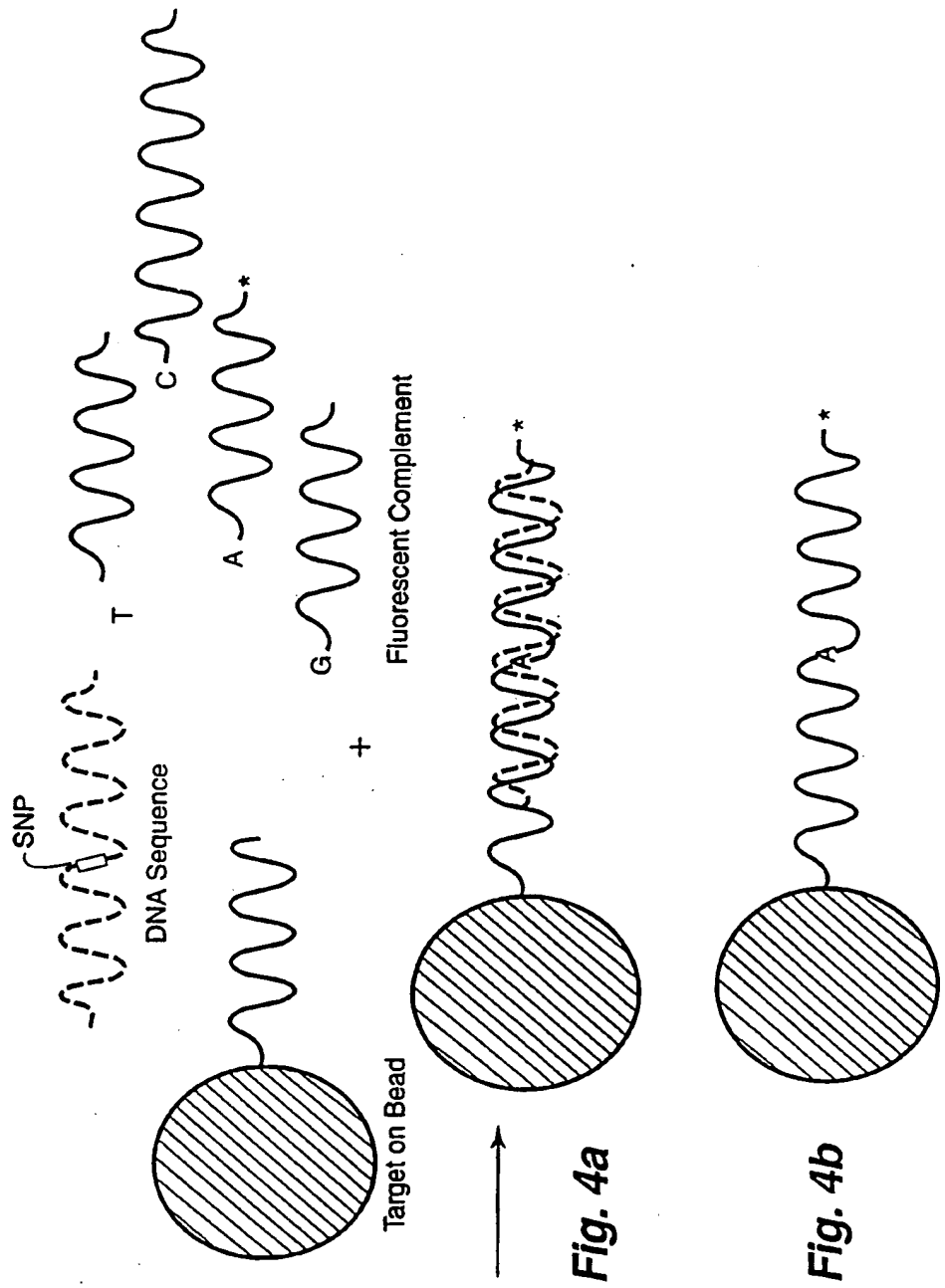

Fig. 4a

Fig. 4b

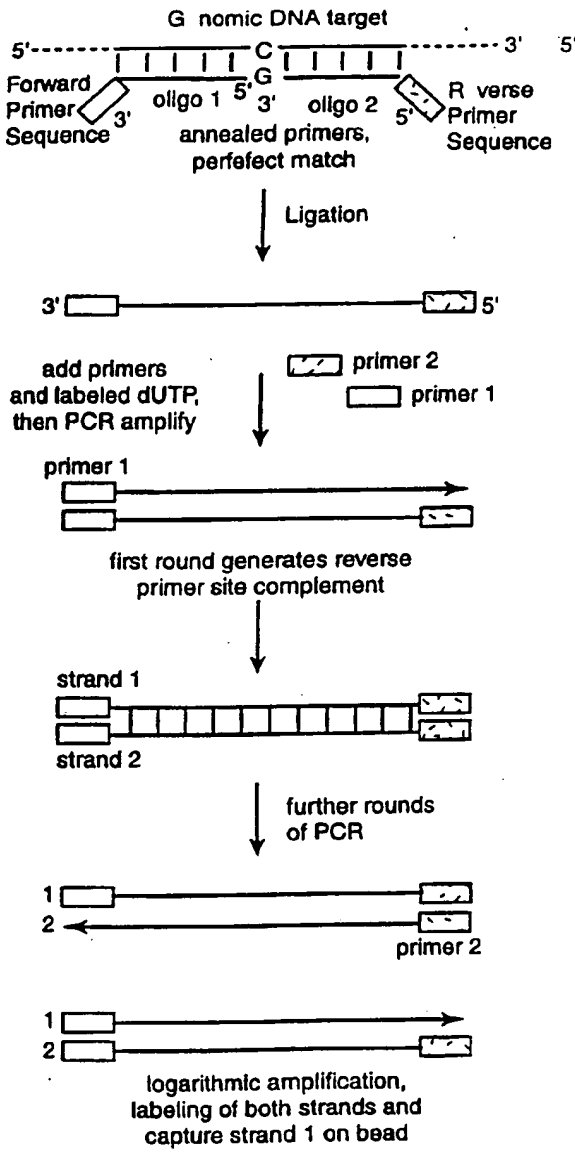

Fig. 5a

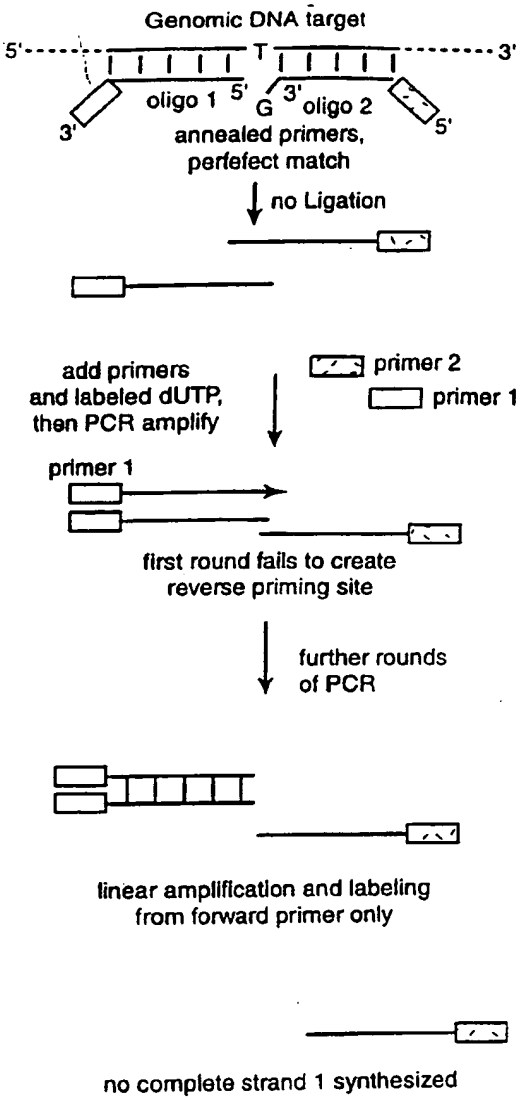

Fig. 5b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23143

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/68; C07H 21/02, 21/04; C12N 15/00
US CL :435/6; 536/23.1, 24.3; 935/76, 77, 78
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1, 24.3; 935/76, 77, 78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LANDEGREN et al. A Ligase-Mediated Gene Detection Technique. Science. 26 August 1988, Vol. 241, pages 1077-1080, see especially Figure 1.	8-27
Y	UGOZZOLI et al. Detection of Specific Alleles by using Allele-Specific Preimer Extension Followed by Capture on Solid Support. GATA. 1992. Vol. 9, No. 4, pages 107-112, see the entire document, but note especially Figure 1.	15-20
Y	US 5,512,439 A (HORNES et al) 30 April 1996, see the entire document.	8-27
Y	US5,629,158 A (UHLEN M.) 13 May 1997, see the entire document.	8-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 05 JANUARY 1999	Date of mailing of the international search report 05 FEB 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ETHAN WHISENANT Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23143

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,770,992 A (VAN DE ENGH et al) 13 September 1988, see the entire document.	8-27
Y,P	US 5,736,330 A (FULTON, R.J.) 07 April 1998, see the entire document.	8-27
Y	BAINS et al. Flow Cytometric Quanitation of Sequence-Specific mRNA in Hemopoietic Cell Suspensions by Primer-Induced in situ (PRINS) Fluorescent Nucleotide Labeling. Experimental Cell Research. 1993, Vol. 208, No. 1, pages 321-326, see the entire document.	15-20
Y	VLIEGER et al. Quantitation of Polymerase Chain Reaction Products by Hybridization-based Assays with Fluorescent, Colorimetric, or Chemiluminescent Detection. Analytical Biochemistry. 15 August 1992, Vol. 205, No. 1, pages 1-7, see the entire document.	8-27
X,P	NOLAN et al. Microsphere-based Assays for Genome Analysis Using Flow Cytometry. American Journal of Human Genetics. 28 October 1997 - 01 November 1997. Vol.61, No. 4, Supplemental. page A241, see the entire abstract.	8-27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23143

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-7
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Page 12 is missing from the application. Page 12 appears to be the page that carries Claims 1-5. Claims 6-7 could not be searched because they are dependent on Claim 1.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/23143

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

USPATFULL, Biosis, Medline, CAplus